Reduced MiR-675 in Exosome in H19 RNA-Related Melanogenesis via MITF as a Direct Target

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H19 non-coding RNA downregulation stimulates melanogenesis in melasma patients. However, its mechanism is unclear. In this study, the potential role of a H19 microRNA, miR-675, in melanogenesis was examined. Real-time PCR using cultured normal human skin keratinocytes, melanocytes, and fibroblasts with or without H19 knockdown showed accompanying changes between expression levels of H19 and those of miR-675 in keratinocytes. MiR-675 was also detected in concentrated culture supernatants and showed expression levels parallel with those of cell lysates. In addition to RNase resistance, FACS analysis showed anti-CD63-positive exosomes in culture supernatants, suggesting miR-675 could be released extracellularly and delivered to neighboring cells without degradation. In western blot analysis, the miR-675 mimic reduced the expression of microphthalmia-associated transcription factor (MITF) and phosphorylation of cAMP-responsive element-binding protein, extracellular signal–regulated kinase and apoptosis signal–regulating kinase, whereas these expressions were increased by the miR-675 inhibitor. Although H19 was not a miR-675 target, luciferase reporter assay showed a direct binding of miR-675 to 3′-untranslated region of MITF. In addition, localized *in vivo* miR-675 overexpression in mouse using a cationic polymer transfection reagent showed reduced mRNA expression levels of MITF, tyrosinase, tyrosine-related protein-1 (Trp-1), and Trp-2. Collectively, the results suggest that miR-675 derived from keratinocytes could be involved in H19-stimulated melanogenesis using MITF as a target of miR-675.

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INTRODUCTION

H19 downregulation is stimulated melanogenesis in melasma patients (Kim *et al.*, 2010). H19 RNA is a 2.3 kb long non-coding RNA (Gabory *et al.*, 2006). An important role of non-coding RNAs is epigenetic regulation of genes. Epigenetic modifications are mainly induced by methylation and acetylation of histones and regulatory factors, DNA methylation, and small non-coding RNAs (reviewed by Bayarsaihan, 2011). As H19 consists of an imprinted cluster with IGF2 and the imprinting control region between the two genes (Jinno *et al.*, 1996; Reese and Bartolomei, 2006), methylation of maternal imprinting control region, which results in simultaneous *H19* gene downregulation and *IGF2* overexpression, has mainly been considered as the mechanism of H19 in tumorigenesis. However, there is no clue as to whether melasma patients are in fact prone to developing tumors. Methylation of maternal imprinting control region has also been variable without a consistent increase in hyperpigmented skin compared with normally pigmented skin in patients, regardless of gender (Kim *et al.*, 2010). These findings suggest that a mechanism other than DNA methylation may be involved in H19 downregulation-induced melanogenesis.

MicroRNAs (miRNAs) are small, 20–24 nucleotides, endogenously expressed non-coding RNAs that regulate gene expression post-transcriptionally by degradation or translational inhibition of target mRNAs. As emerging evidence supports the association between miRNAs and a broad range of pathological conditions, such as cancer, cardiovascular diseases, diabetes, liver diseases, respiratory diseases, psychiatric diseases, neurological diseases, and inflammatory and autoimmune diseases, the discovery of miRNAs is one of the major scientific breakthroughs in the current field of cell biology and medical science (reviewed by Ha, 2011). H19 RNA may give rise to miRNAs. In particular, a 23-nucleotide miRNA, termed miR-675, has been identified in human keratinocytes (Cai and Cullen, 2007). Moreover, cells, which do not normally express H19, transfected with H19 expression plasmid have been observed to express miR-675, suggesting H19 as the origin of miR-675 (Cai and Cullen, 2007). The role of miR-675 has been presented in relation to colorectal cancer (Tsang *et al.*, 2010) and cartilage recovery (Dudek *et al.*, 2010; Steck *et al.*, 2012). The role of miR-675 in mammalian development has been suggested by

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Abbreviations: AKT, apoptosis signal–regulating kinase; CREB, cAMP-responsive element-binding protein; ERK, extracellular signal–regulated kinase; miRNA, microRNA; siRNA, small interfering RNA; Trp-1, tyrosine-related protein-1; UTR, untranslated region

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inhibition of imprinted non-coding RNA expression through an RNA interference mechanism (Cai and Cullen, 2007). An action through a target has also been identified, such as a tumor-suppressor retinoblastoma, which has found to be a direct target of miR-675 (Tsang *et al.*, 2010). In terms of miRNAs in skin pigmentation, miR-25 has been presented to regulate the coat color of alpacas (Zhu *et al.*, 2010). However, no study has so far been reported for the involvement of miR-675 in melanogenesis.

It is also unclear how miR-675 exerts its action on melanocytes if this miRNA originated from keratinocytes (Cai and Cullen, 2007). In fact, H19-associated melanogenesis was more obvious in co-cultures of H19 knockdown keratinocytes and normal melanocytes compared with that of monocultures of H19 knockdown melanocytes in a previous study (Kim *et al.*, 2010). Cell–cell communication may occur through several ways, through receptors, direct cell–cell contact, and cell–cell synapses. In addition, exosomes, which are small membrane vesicles of endocytic origin released by many cells into the extracellular environment, have been identified to contain large amounts of intact and functional small RNAs, including miRNAs. The transfer of exosome-derived miRNAs to recipient cells has suggested to be a mechanism allowing gene-based communication between mammalian cells (Valadi *et al.*, 2007).

Although H19 downregulation has been identified to be involved in skin pigmentation of melasma, H19 is a non-coding RNA. In this study, the role of miRNAs, particularly that of miR-675, was examined as a potential mechanism behind H19 RNA-related skin pigmentation. For the purpose, we examined whether miR-675 is associated with H19, the type of skin cells are involved in H19-related miR-675 expression, and how miR-675 is involved in melanogenesis. As miRNAs degrade or translationally inhibit their target mRNAs, microphthalmia-associated transcription factor (MITF) was chosen as a candidate target from a microarray analysis using miR-675 and database for miRNA target prediction (miRDB), and this association was also experimentally validated. The results showed that miR-675 is released from keratinocytes as exosomes and regulate H19 downregulation-induced melanogenesis through MITF as a direct target of miR-675.

RESULTS

H19 knockdown reduces miR-675 expression levels in keratinocytes, but not in melanocytes

H19 RNA expression was examined in cultured normal human epidermal keratinocytes, melanocytes, and in dermal fibroblasts. Absolute expression levels decreased in order in keratinocytes, melanocytes, and fibroblasts. The levels in melanocytes and fibroblasts were much lower than those in keratinocytes (Figure 1a). MiR-675 expression was also examined in keratinocytes, melanocytes, and fibroblasts in the absence or presence of H19 small interfering RNA (siRNA). In keratinocytes, H19 knockdown significantly ($P<0.05$) reduced the expression levels of miR-675 (Figure 1b). However, H19 levels did not have a significant association with miR-675 expression levels in melanocytes (Figure 1c) and fibroblasts (data not shown).

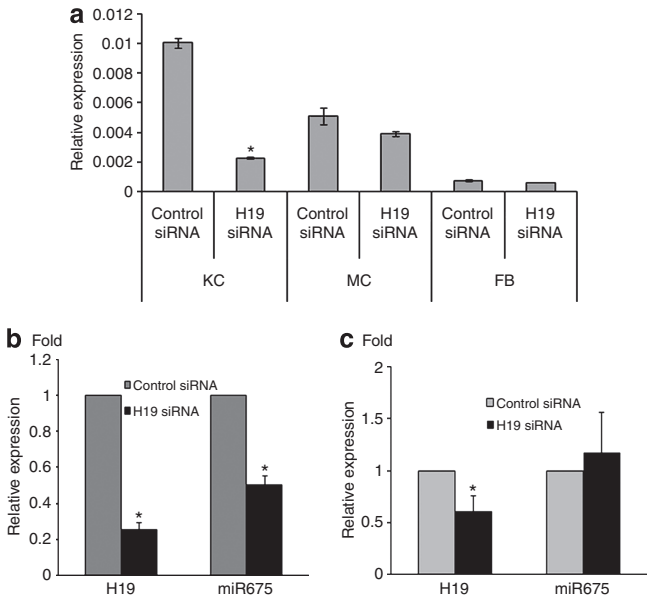

Figure 1. miR-675 expression levels in keratinocytes and melanocytes with or without H19 knockdown. (**a**) Absolute expression levels of H19 in keratinocytes, melanocytes, and fibroblasts with or without H19 knockdown using H19 small interfering RNA (siRNA). (**b**, **c**) Real-time PCR results for relative ratios of H19 RNA and miR-675 expression levels in cultured normal human epidermal keratinocytes (**b**) and melanocytes (**c**) with or without H19 knockdown using H19 siRNA. *$P<0.05$, data represent mean ± SD of five independent experiments. FB, fibroblast; KC, keratinocyte; MC, melanocyte.

MiR-675 could be released from cultured keratinocytes into culture media as exosomes

Expression levels of miR-675 correlated with those of H19 in keratinocytes (Figure 1b), but not for melanocytes (Figure 1c) nor fibroblasts (data not shown). As H19 knockdown in keratinocytes increases melanogenesis and melanosome transfer from co-cultured normal melanocytes (Kim *et al.*, 2010), it could be expected that miR-675 would induce similar results in keratinocytes. However, it is unclear how miR-675 from keratinocytes can influence melanocytes. Thus, we examined whether miR-675 could be released extracellularly from keratinocytes to exert a direct action on neighboring skin cells. Real-time PCR showed that expression levels of miR-675 in cell lysates paralleled those in culture supernatants (Figure 2a). Treatment of concentrated culture supernatants with RNase did not reduce miR-675 expression levels (Figure 2b). FACS analysis of the concentrated structures in the supernatant, which were adsorbed with latex beads, then stained with anti-CD63 antibody, also revealed that anti-CD63-positive exosomes were present in concentrated culture supernatants (Figure 2c).

miR-675 mimic reduces, whereas inhibitor stimulates melanogenesis

Production of miR-675 was dependent on H19 levels in keratinocytes (Figure 1b). Intracellular miR-675 was also present in culture media as exosomes in keratinocytes (Figure 2a–c), suggesting that miR-675 could be delivered by exosomes to neighboring melanocytes and/or fibroblasts.

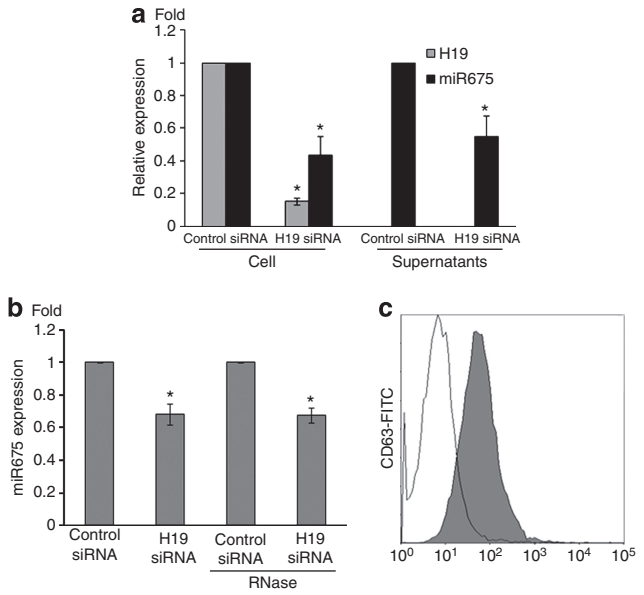

Figure 2. miR-675 expression in cell lysates versus supernatants of cultured keratinocytes. (**a**) Real-time PCR results for relative ratios of H19 RNA and miR-675 expression levels in cell lysates and supernatants of cultured normal human epidermal keratinocytes, with or without H19 knockdown. (**b**) Real-time PCR results for relative ratios of miR-675 expression levels in exosomes purified from the culture supernatants (described in Materials and Methods section), with or without RNase treatment. (**c**) FACS analysis results using anti-CD63 antibody in purified exosomes (filled trace) or negative control (open trace), which were absorbed onto latex beads. *$P<0.05$, data represent mean ± SD of five independent experiments. siRNA, small interfering RNA.

Therefore, the miR-675 mimic or inhibitor was transfected into keratinocytes or fibroblasts, which were co-cultured with normal melanocytes, or directly into melanocytes. Subsequently, the effect of miR-675 on melanogenesis was examined. The miR-675 mimic significantly ($P>0.05$) reduced the expression levels of tyrosinase in both co-cultured systems (Figure 3a and b) and melanocyte monoculture systems (Figure 3c), whereas the inhibitor increased these expression levels (Figure 3a–c). In melanocyte monocultures, the effects of miR-675 on the expression of tyrosinase, tyrosine-related protein-1 (Trp-1), and Trp-2 were dose dependent (Figure 3c). Western blot analysis to examine signaling pathways involved in melanogenesis showed that the miR-675 mimic in melanocytes significantly ($P>0.05$) reduced the phosphorylation of cAMP-responsive element-binding protein (CREB), extracellular signal–regulated kinase (ERK), apoptosis signal–regulating kinase (AKT), and nuclear factor kappa B, whereas the knockdown induced opposite results (Figure 3d). Protein kinase C phosphorylation was not changed by either the miR-675 mimic or the inhibitor (Figure 3d).

MiR-675 transfection results in no significant change in H19 expression levels in keratinocytes, melanocytes, and fibroblasts

As miR-675 has been suggested to inhibit the expression of imprinted non-coding RNA through an RNA interference mechanism (Cai and Cullen, 2007), we examined whether miR-675 could reduce H19 expression levels in the same manner as H19 siRNA did (Kim *et al.*, 2010). As miR-675 could be delivered to neighboring cells as exosomes, the effect of miR-675 on H19 expression levels was examined in keratinocytes as well as melanocytes and fibroblasts. In contrast to previous findings with regards to H19 siRNA, miR-675 transfection using the mimic did not cause significant changes to H19 expression levels in keratinocytes, melanocytes, or fibroblasts (Figure 4a–c, respectively).

MiR-675 inhibits MITF expression by targeting its 3′-untranslated region (UTR)

The finding that miR-675 did not reduce H19 expression levels (Figure 4a–c) indicates that H19 is not a direct target of miR-675. MITF was suggested as a potential target of miR-675 by the microarray data obtained from cells treated with and without the miR-675 mimic, showing >1.5-fold reduction (Supplementary Table S1 online), and by MiRDB (http://mirdb.org). Therefore, MITF was examined as a potential direct target of miR-675, using cloned full-length 3′-UTR and deletion mutant of MITF. In fact, 7 bp of complementary sequences were found between miR-675 and 3′-UTR of MITF mRNA (Figure 5a). Luciferase activity was reduced significantly ($P<0.05$) in both 293T cell line (Figure 5b) and cultured normal human melanocytes (Figure 5c), which were transfected with pMIR-MITF (full length) and miR-675 but not with the other combination of pMIR-REPORT/pMIR-MITF (full length) and negative control miRNA/miR-675. In case of deletion mutant MITF, miR-675 did not significantly reduce luciferase activity in the 293T cell line or in melanocytes (Figure 5b and c, respectively). In addition, the miR-675 mimic significantly ($P<0.05$) and dose-dependently reduced expression levels of MITF mRNA and protein, whereas the inhibitor significantly ($P<0.05$) and dose-dependently increased the respective expression levels (Figure 5d).

In vivo delivery of miR-675 mimic reduced melanogenesis in mouse skin

The effect of miR-675 on melanogenesis and association between miR-675 and MITF was also examined in the biopsied skin of C57BL/6J mice injected with the miR-675 3p mimic or negative control with a cationic polymer transcription reagent, *in vivo*-jetPEI (Polyplus-transfection SA, Illkirch, France). Transfer of miR-675 3p into mouse skin reduced the mRNA expression of MITF, tyrosinase, Trp-1, and Trp-2 (Figure 6a). Immunohistochemistry also detected reduced tyrosinase and MITF in the miR-675 3p transferred skin compared with the normal control skin (Figure 6b and c, respectively).

DISCUSSION

H19 RNA gives rise to a group of miRNAs, of which miR-675 has been identified in human keratinocytes (Cai and Cullen, 2007) and colon cancer cell lines (Tsang *et al.*, 2010). In this study, H19 expression was examined in melanocytes and fibroblasts, as well as in keratinocytes (Figure 1a). H19 expression has previously been identified in fibroblasts (Hayashida *et al.*, 1997; Stuhlmüller *et al.*, 2003). As expected, miR-675 expression was identified in melanocytes (Figure 1c) and fibroblasts (data not shown). Nonetheless, an accompanied change between H19 and miR-675 was

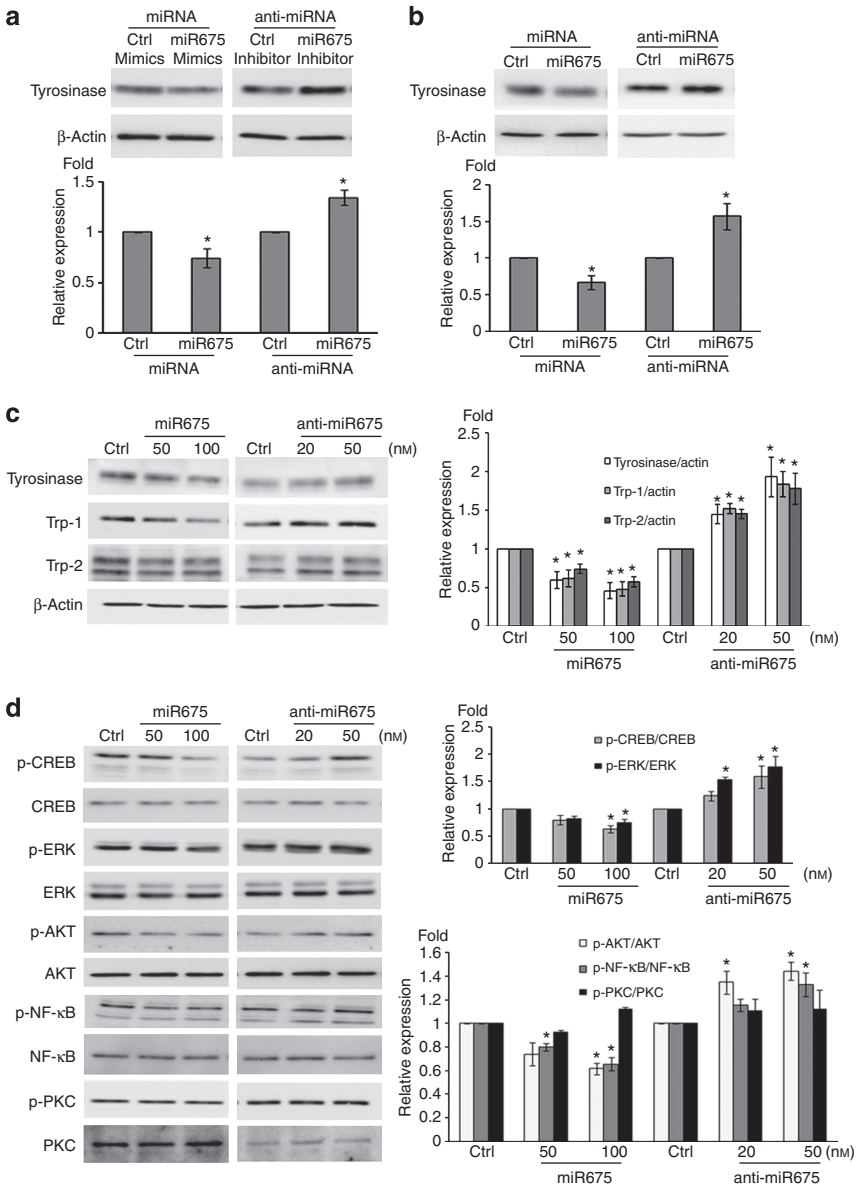

Figure 3. Effect of miR-675 mimic and inhibitor on melanogenesis. (**a**, **b**) Western blot analysis of tyrosinase expression in melanocytes co-cultured with keratinocytes (**a**) or fibroblasts (**b**), which were transfected by either the miR-675 mimic or inhibitor. (**c**, **d**) Western blot results for the expression of tyrosinase, Trp-1, Trp-2 (**c**) and signaling molecules involved in melanogenesis (**d**) in melanocyte monocultures transfected by either the miR-675 mimic or inhibitor. *$P<0.05$, data represent mean ± SD of five independent experiments. AKT, apoptosis signal–regulating kinase; anti-miR, microRNA hairpin inhibitor; CREB, cAMP-responsive element-binding protein; ctrl, negative control; ERK, extracellular signal–regulated kinase; miRNA, microRNA; NF-κB, nuclear factor kappa B; PKC, protein kinase C; Trp-1, tyrosine-related protein-1.

required to identify whether miR-675 was involved in melanogenesis. As H19 RNA downregulation is known to occur in melanogenesis (Kim *et al.*, 2010), miR-675 expression with or without H19 knockdown using siRNA was examined in keratinocytes, melanocytes, and fibroblasts cultured from normal human skin. The result that expression levels of miR-675 paralleled the expression levels of H19 RNA only in keratinocytes (Figure 1b) suggested that miR-675 originates from keratinocytes to mediate its role in melanogenesis.

Melanogenesis occurs in melanocytes. Therefore, transference from keratinocytes to melanocytes could provide a mechanism for miR-675 to inhibit melanogenesis. In fact, concurrent presence of miR-675 in both cell lysates and culture supernatants of keratinocytes (Figure 2a) suggested extracellular release of miR-675. However, the presence of extracellular miRNA may not necessarily signify a functional cascade. A discrete mode of delivery to melanocytes and other neighboring cells is required to prevent degradation and to maintain the function of miR-675. Exosomes, which are vesicles of endocytic origin released into the extracellular environment, have been known to mediate a potential mode of intercellular communication (Van Niel *et al.*, 2006). In addition, exosomes have been known to contain mRNAs and miRNAs (Valadi *et al.*, 2007; da Silveira *et al.*, 2012).

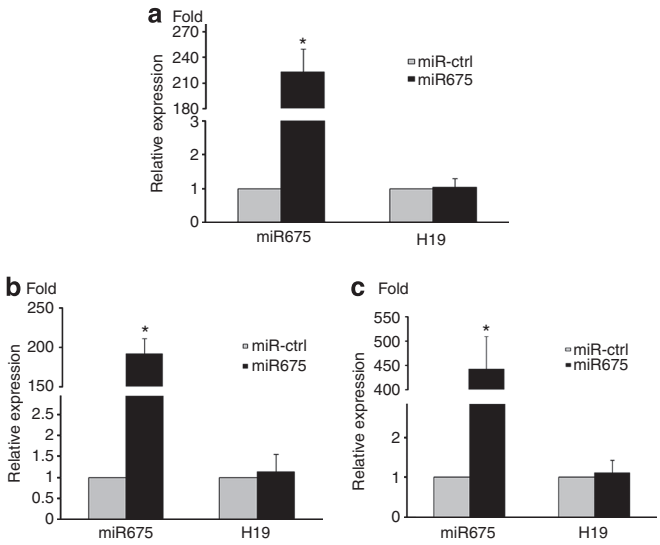

Figure 4. No significant effect of miR-675 on H19 expression levels in keratinocytes, melanocytes, and fibroblasts. Real-time PCR results for relative ratios of H19 RNA expression levels in keratinocytes (**a**), melanocytes (**b**), and fibroblasts (**c**) with or without miR-675 transfection. *$P<0.05$, data represent mean ± SD of five independent experiments.

Exosome-derived mRNAs and miRNAs have been shown to be resistant to RNase treatment and be functional after transfer to recipient cells (Valadi *et al.*, 2007). This study also showed that RNase treatment did not significantly decrease miR-675 expression levels in keratinocyte culture supernatants (Figure 2b). This result suggests that miR-675 was confined inside a certain structure. In fact, FACS analysis showed that the latex beads coated with a certain structure were reacted with anti-CD63 antibody (Figure 2c), which indicated the presence of exosomes (Clayton *et al.*, 2001; Valadi *et al.*, 2007; da Silveira *et al.*, 2012) in culture supernatants. To the best of our knowledge, our experimental results showing an involvement of miRNA within exosomes in intercellular transfer from keratinocytes to melanocytes are previously unreported.

As miR-675 can be transferred to neighboring cells through exosomes (Figure 2b and c), miR-675 may exert its action in melanocytes directly as well as indirectly through miR-675 containing keratinocytes or fibroblasts. Although no study has reported the involvement of miR-675 in melanogenesis, this study suggests that miR-675 inhibit melanogenesis in these three common types of skin cells (Figure 3a–c). MITF-M is the most critical transcriptional factor required for the regulation of melanocyte function. CREB in cAMP pathway is a molecule involved in activation of MITF-M (Widlund and Fisher, 2003). To examine the mechanisms involved in the regulation of melanogenesis, CREB was examined with protein kinase C and AKT/protein kinase B (Gordon and Gilchrest, 1989; Park *et al.*, 2006). In this part of the experiment, the miR-675 mimic decreased phosphorylations of CREB, ERK, AKT, and NF-κB, whereas the miR-675 inhibitor increased the respective phosphorylations (Figure 3d). This result suggested a regulatory role of cAMP, ERK, and AKT pathways in miR-675-related melanogenesis, regardless of cell types.

Parallel changes were identified between miR-675 and H19 in keratinocytes (Figure 1b). Nonetheless, miR-675 was examined for its ability to inhibit H19 levels because reductions in H19 RNA expression levels by H19 siRNA are known to be associated with melanogenesis (Kim *et al.*, 2010). In contrast to H19 siRNA, however, miR-675 did not reduce H19 expression levels (Figure 4a–c), suggesting that H19 is not a direct target of miR-675. On the other hand, complementary sequences between 7 bp of miR-675 and 3′-UTR of MITF (Figure 5a) suggested this site to be the only miR-675 site in the transcript. Moreover, luciferase reporter assays (Martin *et al.*, 2006) showed that full-length 3′-UTR, but not deletion mutant, of MITF reduced MITF expression in two kinds of cells including normal human melanocytes containing miR-675 mimic (Figure 5b and c). This result indicates a direct binding of miR-675 to the 3′-UTR of MITF.

To confirm the involvement of miR-675 in melanogenesis inhibition, *in vitro* results requires validation in an *in vivo* model. As melasma is a hyperpigmented skin disorder affecting particular locations, but neither the entire skin nor other organs, localized percutaneous delivery of the miRNAs is a potential mechanism worth investigating. In this study, repeated subepidermal injection (Murase *et al.*, 2009) of the mouse miR-675 mimic or negative control with a cationic polymer transfection reagent induced localized miR-675 overexpression *in vivo*, although the miR-675 overexpression levels were quite different across various specimens. In addition, mRNA expression levels of miR-675 were reciprocal with those of MITF, tyrosinase, Trp-1, and Trp-2 in miR-675-overexpressed skin specimens (Figure 6a). Reciprocal expressions were also identified in immunohistochemistry using anti-tyrosinase and anti-MITF antibodies in miR-675-overexpressed skin specimens (Figure 6b and c, respectively). Although the discrepancies among *in vivo* results may be caused by a lack of organ or cell specificity in gene delivery using non-viral vectors (Hashida *et al.*, 2001), these *in vivo* results help to confirm the role of miR-675 in H19 RNA downregulation-induced melanogenesis.

MATERIALS AND METHODS

Normal human epidermal cell culture

Adult skin specimens obtained from cesarean sections and circumcisions were used for establishing cells in culture. The epidermis was separated from the dermis, and then suspensions of individual epidermal cells were prepared. Keratinocytes were suspended in EpiLife Medium (#M-EPI-500-CA; Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract, bovine insulin, hydrocortisone, human epidermal growth factor, and bovine transferrin (#S-001-5; Invitrogen). Keratinocytes from passages 3 or 4 were used in these experiments. Melanocytes were suspended in medium 254 (Invitrogen) supplemented with bovine pituitary extract, fetal bovine serum, bovine insulin, hydrocortisone, basic fibroblast growth factor, bovine transferrin, heparin and phorbol 12-myristate 13-acetate (Invitrogen). Melanocytes at passage numbers between 7 and 15 were used for these experiments. For fibroblast culture, individual dermal cells were suspended in DMEM (Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco/BRL), 100 U ml^{-1} penicillin

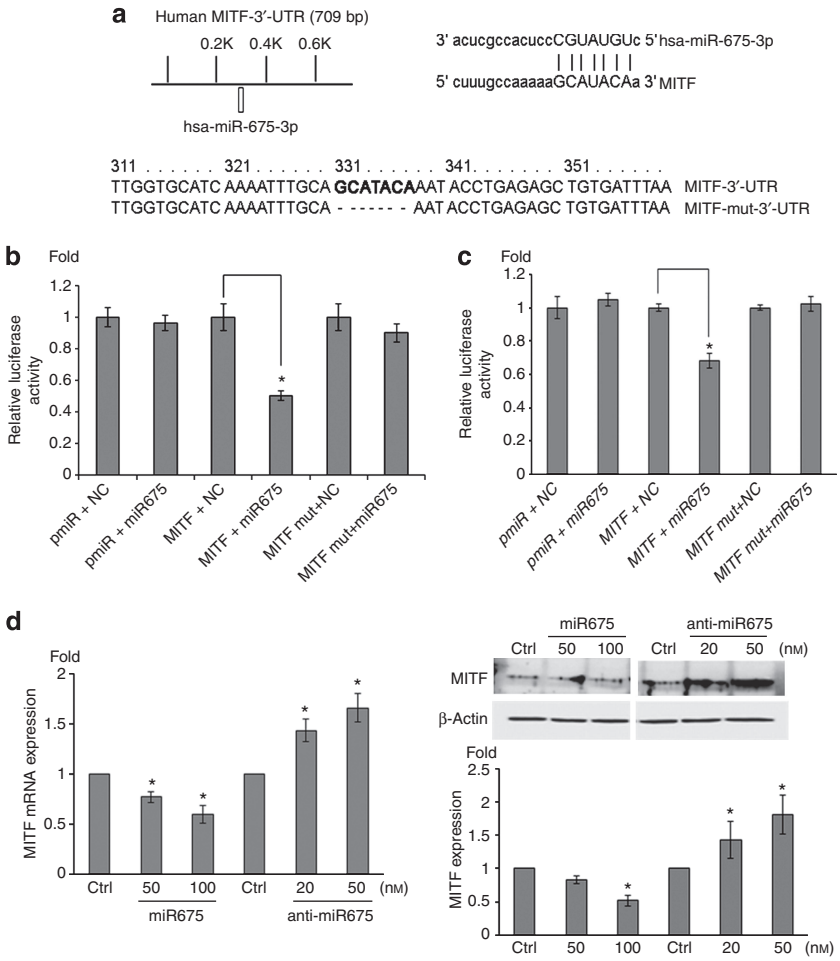

Figure 5. Microphthalmia-associated transcription factor (MITF) as a direct target of miR-675. (**a**) Complementary sequences between miR-675 and 3′-untranslated region (UTR) of MITF mRNA. (**b**, **c**) Relative ratios of luciferase activity in the 293T cell line (**b**) and cultured normal human melanocytes (**c**), which were transfected by pMIR-MITF full length (MITF) or pMIR-MITF deletion mutant (MITF mut), with or without miR-675. (**d**) Real-time PCR and western blot analysis of MITF mRNA and protein expression in melanocytes treated with miR-675 mimic (miR675) or inhibitor (anti-miR675). *$P<0.05$, data represent mean ± SD of five independent experiments. The bold and lowercase letters denote 7bp of complementary sequence between miR-675 and 3′URT of MITF.

(Gibco/BRL), and 0.1 mg ml^{-1} streptomycin (Gibco/BRL). Fibroblasts at passages between 5 and 10 were used.

Real-time reverse transcriptase–PCR analysis

The expression of mature miRNAs was assayed using Taqman MicroRNA Assays (Applied Biosystems, Foster, CA) specific for hsa-mir-675. TaqMan MicroRNA assays were used for miRNA quantification by real-time reverse transcriptase–quantitative PCR. Real-time quantitative PCR was performed with the Light Cycler480 probe master mix (Roche, Mannheim, Germany), and U18 was used as a housekeeping gene for normalization (Applied Biosystems). The levels of mRNAs relative to glyceraldehyde-3-phosphate dehydrogenase were measured by quantitative real-time PCR using the Light Cycler real-time PCR (Roche).

Exosome purification

Exosomes were prepared from the supernatant of keratinocytes by differential centrifugation, as previously described (Hadi *et al.*, 2007). Briefly, cells were harvested and centrifuged at 500 *g* for 10 minutes to eliminate cells and then centrifuged again at 16,500 *g* for 20 minutes, followed by filtration through a 0.22 μm filter. Exosomes were pelleted by ultracentrifugation at 120,000 *g* for 70 minutes.

Flow cytometry

For FACS analysis, exosomes from keratinocytes were adsorbed onto 4 μm aldehyde-sulphate latex beads (Interfacial Dynamics, Tualatin, OR) and incubated with CD63- FITC antibody (Beckman Coulter, Marseille, France). The cells were then washed thoroughly with phosphate-buffered saline and prepared for flow cytometry. Labeled cells were analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter, Hialeah, FL) using CXP software (Beckman Coulter).

H19 knockdown

For the H19 downregulation, melanocytes and keratinocyte cultured in six-well plates were transfected with two different stealthTM siRNAs (Stealth 243 and Stealth 472) and a negative control stealth siRNA (Invitrogen), using TransIT-siQUEST transfection reagent (Mirus, PanVera, Madison, WI) and 80 nM siRNA, according to the manufacturer's instructions.

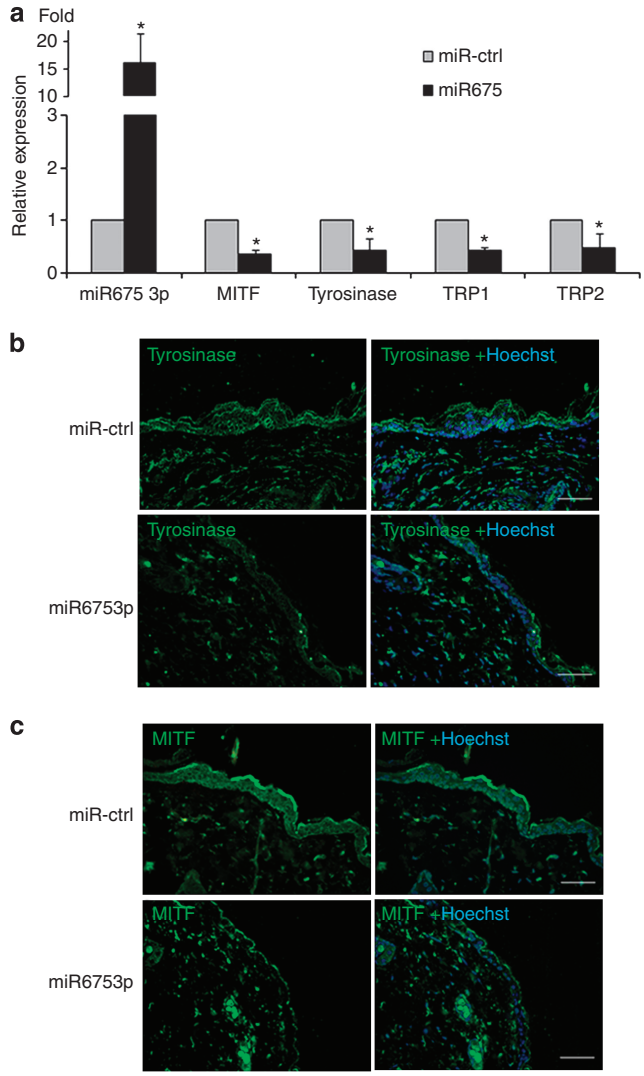

Figure 6. *In vivo* delivery of miR-675 mimic on melanogenesis in mouse skin. (**a**) Real-time PCR results for relative ratios of miR-675, microphthalmia-associated transcription factor (MITF), tyrosinase, tyrosine-related protein-1 (Trp-1), and Trp-2 expression levels in mouse skin injected subepidermally with mouse miR-675 3p or negative control miRNA using a cationic polymer transfection reagent. *$P<0.05$, data represent mean ± SD of five independent experiments. (**b**, **c**) Immunohistochemical staining of mouse skin injected mouse miR-675 3p or negative control miRNA using anti-tyrosinase (**b**) and anti-MITF antibodies (**c**). Nuclei were counterstained with Hoechst 33258 (bar = 0.05 mm).

Transfection of miRNA mimics and inhibitors

Cells were transfected with negative control miRNA mimics (Dharmacon Research, Lafayette, CO), human miR-675 mimics (Dharmacon Research), negative control hairpin inhibitor, or miR-675 hairpin inhibitor. The TransIT-siQUEST transfection reagent (Mirus) was used for transfection according to the manufacturer's protocol.

Microarray

Total RNA from cells cultured under each condition was isolated using QuickGene RNA cultured cell kit S (Life Science, Tokyo, Japan), according to the manufacturer's instructions. Arrays were prepared, hybridized, and scanned at the local authorized Illumina array service provider (MACROGEN, Seoul, South Korea). Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX) to yield biotinylated complementary RNA, according to the manufacturer's instructions. In all, 750 ng of labeled complementary RNA, samples were hybridized to each human HT-12 expression v.4 bead array for 16–18 hours at 58 °C, according to the manufacturer's instructions (Illumina, San Diego, CA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array reader confocal scanner according to the manufacturer's instructions. The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2009.2; Gene Expression Module v1.5.4). Array data were filtered by detection P-value <0.05 (similar to signal to noise) in at least 50% samples (we applied a filtering criterion for data analysis; higher signal value was required to obtain a detection P-value <0.05). Selected gene signal value was transformed by logarithm and normalized by quantile method.

Western blot analysis

Equal amounts of extracted proteins (20 μg) were resolved using 10% SDS-PAGE and were then transferred to nitrocellulose membranes. The membranes were incubated with antibodies to phospho-ERK, ERK, phospho-CREB, CREB, phospho-AKT, AKT, phospho-NF-κB, NF-κB, phospho-protein kinase C, protein kinase C, beta-catenin (rabbit polyclonal; Cell Signaling Technology, Beverly, MA), and tyrosinase (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), and further with anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology), followed by treatment with an enhanced chemiluminescence solution (Thermo, Rockford, IL). Signals were captured on an Image Reader (LAS-3000; Fuji Photo Film, Tokyo, Japan). To monitor the amount of protein loaded in each lane, the membranes were reprobed with mouse monoclonal anti-β-actin antibody (Sigma, St Louis, MO) and processed as described above. Protein bands were analyzed by densitometry.

Immunohistochemistry

For immunofluorescence staining, after deparaffinization and rehydration, the sections were preincubated with 3% BSA. For double staining, the cultured cells or sections were reacted sequentially with anti-MITF, tyrosinase antibody and 1:200 Alexa Fluor-labeled goat anti-rabbit IgG (488; Molecular Probes, Eugene, OR), and then with anti-c-kit and Alexa Fluor-labeled mouse anti-mouse IgG (594; Molecular Probes). Nuclei were counterstained with Hoechst 33258 (Sigma). The stained specimens were evaluated using an image analysis system (Dp Manager 2.1; Olympus Optical, Tokyo, Japan).

Luciferase activity assay

Full-length human MITF 3′-UTR was amplified by PCR using the forward primer 5′-AAAGCTGCGCACTAGTGAATGCAGAGAGAG GAGAAG-3′ (MITF-3UTR-F) and the reverse primer 5′-ATCCTT TATTAAGCTTTTGGATTTGCAAATGACT-3′ (MITF-3UTR-R). The amplified 3′-UTR was cloned downstream of the luciferase gene in

pMIR-REPORT luciferase vector (Ambion). This construct, named pMIR-MITF, was used for transfection in melanocytes and 293T. The deletions were generated with the predicted target sites of MITF 3′-UTR using the QuickChange site-directed mutagenesis kit (Stratagene, Lo Jolla, CA) according to the manufacturer's instructions. Cells were cultured in six-well plates, and each was transfected with pMIR-MITF or pMIR-mutant together with pRL-TK vector (Promega, Madison, WI) containing Renilla luciferase, and 50 nM of miR-675 or negative control (Dharmacon Research). Transfection was done using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, firefly and Renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay (Promega). Transfection was performed in triplicates, and all experiments were repeated three times.

Injection of miRNA into mouse

C57BL/6J female mice were used. The animal care and use committee of the research institute at Dongguk University Hospital approved all described studies. Ears and back of each mouse were injected subepidermally with 5 μg of mouse miR-675 3p or negative control miRNA with a cationic polymer transfection reagent, *in vivo*-jetPEITM (Polyplus-transfection SA). In total, they were administrated with miRNA injection three times at 24-hour intervals.

Statistical analysis

Statistical analysis of experimental data was performed using the Student's *t*-test. A *P*-value <0.05 was considered to be statistically significant. All results are presented as mean ± SD of the combined data from repeated experiments.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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